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Detection of Hepatitis G Virus RNA in Patients With Hepatitis B, Hepatitis C, and Non-A-E Hepatitis by RT-PCR Using Multiple Primer Sets

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Hepatitis G virus(HGV)/GB virus C(GBV-C) is a newly identified virus associated with human hepatitis. The preliminary prevalence studies of HGV infection in Japan were entirely based on the detection of HGV RNA by RT-PCR. However, the selection of the different primer sets in such assay may influence sensitivity of the test because of the extensive genetic heterogeneity of HGV, and influence the estimation of the prevalence of HGV. To address this potential problem, we designed two primer sets from well conserved domains in the 5′NC and NS5 regions of HGV genome, and tested them together with the NS3-derived primer set in RT-PCR for their ability to detect HGV RNA in serial dilution of synthetic viral RNA templates. Subsequently, we used these three primer sets to detect HGV RNA in the sera of 371 Japanese patients with hepatitis B, hepatitis C, and non-A-E hepatitis. The results indicated that the primer set derived from the 5′NC region appeared to be most effective in detecting HGV RNA. The results also showed that only two out of the 126 patients (1.6%) with non-A-E hepatitis were positive for HGV RNA although the RNA were detected more frequently in patients with hepatitis B (2/38; 5.3%) and hepatitis C (17/207; 8.2%), suggesting that HGV is not a common causative agent for non-A-E hepatitis in Japan. ***J. Med. Virol. 52:385–390, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: GB virus C; HGV; HCV; HBV carrier

INTRODUCTION

Recently, new presumed hepatitis agents, which were designated GB virus C (GBV-C) and hepatitis G virus (HGV), were independently described by two research teams [Linnen et al., 1996; Simons et al., 1995]. Both GBV-C and HGV are positive strand RNA viruses and are members of the *Flaviviridae* family. Sequence analyses have revealed that GBV-C and HGV are highly homologous in nucleotide and deduced amino acid sequences (identities of 85% and 95%, respectively), suggesting that the two represent closely related variants of the same virus [Alter, 1996; Pessoa et al., 1996].

GBV-C/HGV RNA has been found in patients with acute or chronic hepatitis, fulminant hepatitis, patients on hemodialysis maintenance, intravenous drug users (IVUDs), and blood donors from around the world [Aikawa et al., 1996; de Lamballerie et al., 1996; Leary et al., 1996a; Linnen et al., 1996; Masuko et al., 1996; Yoshiba et al., 1995]. The prevalence rates for GBV-C/HGV infection ranged from approximate 1% in blood donors to 16–39% in IVUDs and patients diagnosed with non-A-E hepatitis [Aikawa et al., 1996; Dawson et al., 1996; Fiordalisi et al., 1996; Leary et al., 1996a; Linnen et al., 1996; Orito et al., 1996; Schreier et al., 1996]. Among these observations, it was found that the prevalence of HGV among Japanese patients on dialysis (3.1%) is significantly lower than those in the U.S. or Europe (19–58%) [Alter, 1996; de Lamballerie et al., 1996; Masuko et al., 1996; Sampietro et al., 1996]. The frequency of infection of HGV in patients with non-A-E fulminant hepatitis was also different between Japan (12–37.5%) and Germany (50%) [Heringlake et al., 1996; Tameda et al., 1996; Yoshiba et al., 1996]. However, the prevalence studies performed in the U.S. and Europe were based on detection of HGV RNA in serum by reverse transcription polymerase chain reaction (RT-PCR) with the primers derived from 5′NC, NS3, or NS5 regions of HGV genome, whereas those in Japan were mainly based upon utilizing the NS3-derived primers [Yoshiba et al., 1995]. This raised the possibility that the mismatch between primer and template in the latter assay may have caused underestimation of the prevalence of HGV infection in Japan [Sampietro et al., 1996], because the NS3 region was found to have considerable degrees of sequence divergence (10–30%)

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TABLE I. Prevalence of HGV Infection in Japanese Patients With Various Hepatitis

Diagnosis		Number of samples tested	HGV positive (%)
Non-A-E-hepatitis	Fulminant	8	1 (12.5)
	Acute	47	0 (0)
	Chronic	44	1 (2.3)
	HCC[a]	27	0 (0)
Hepatitis B	Carrier	38	2 (5.3)
Hepatitis C	Chronic	197	16 (8.1)
	HCC	10	1 (10)

[a]HCC, hepatocellular carcinoma.

among different HGV strains [Pickering et al., 1997; Shao et al., 1996]. To investigate the effectiveness of different primer sets for detecting HGV RNA in RT-PCR assay and to estimate the true prevalence of HGV infection in Japanese patients with hepatitis B, hepatitis C, and non-A-E hepatitis, we designed two primer sets from highly conserved domains in the 5′NC and NS5 regions of HGV genome, and compared them to the NS3-derived primer set which was previously used widely in Japan for their ability to detect HGV RNA in serial dilution of synthetic viral RNA templates. Moreover, we utilized these three primer sets to detect HGV-RNA in the sera of 371 Japanese patients with various types of hepatitis including hepatitis B, hepatitis C, and non-A-E hepatitis.

MATERIALS AND METHODS

Serum Samples

Serum samples were obtained from 126 patients with non-A-E hepatitis, 207 patients with hepatitis C, and 38 HBV carriers (Table I). HBV carriers were defined as the patients having hepatitis B surface antigen (HBsAg; Abbott Laboratories, Abbott Park, IL). Hepatitis C was diagnosed based on the presence of either serum antibodies to HCV (Anti-HCV; Abbott Laboratories) or serum HCV RNA detected by PCR with primers from the highly conserved 5′ noncoding region [Bukh et al., 1992]. Non-A-E hepatitis was defined as those cases that were negative for markers of HAV, HBV, HCV, and HEV, as well as negative for other possible causes of hepatitis (alcoholism, autoimmune diseases, hepatotoxic drugs, and hematochromatosis). Fulminant, acute and chronic hepatitis, and hepatocellular carcinoma were diagnosed based on history, laboratory data, and histological finding.

All sera investigated here were collected at the time of entry into the hospital, and stored at −40°C until RT-PCR analysis.

Oligonucleotide Primers

Three sets of primers deduced from highly conserved regions of HGV genome were used in this study (Table II). Sets I and III were designed from the highly conserved domains within the 5′NC and NS5 regions of HGV genome. Set II from the putative NS3 region was the same as that described by Yoshiba et al. [1995].

Synthetic HGV RNA Template

As described previously [Shao et al., 1996], using the HGV specific primers sw and aNS5, a large cDNA fragment (8609 bp) which nearly covers the whole genome of HGV of a Japanese patient (Iw) with non-A-E hepatitis, was amplified by long reverse transcription-PCR (long RT-PCR). Briefly, RNA was extracted from 200 μl of serum and reverse transcribed to cDNA with SuperScript II Reverse Transcriptase (GIBCO, Gaithersburg, MD) and primer aNS5. The resulting cDNA was added into a long PCR reaction mix (LA PCR kit, ver.2, TaKaRa, Osaka, Japan). Long PCR was performed in the thermocycler PE2400 (Perkin-Elmer, Branchburg, CA) for 32 cycles, each cycle consisting of denaturation at 98°C for 15 sec, primer annealing and extension at 68°C for 10 min. The PCR product was subsequently gel purified and inserted into pT7blue T-vector (Novagen, Madison, WI) according to the manufacturer's recommendation, and then subcloned to pGEM-3zf(+) vector (Promega, Madison, WI).

The plasmid pGEM-GIw, which contains the large HGV cDNA fragment (amplified above), was linearized with Xba I and transcribed in vitro with T7 RNA polymerase (Promega) in order to prepare the HGV genomic RNA molecules. The reaction mixture was then treated with Proteinase K and DNase I. The synthetic HGV RNA was purified with CHROMA SPIN-100+DEPC-H2O Columns (CLONTECH Co.; Palo Alto, CA). The concentration of RNA was detected by measuring absorbance at 260 nm. The purity and molecular size were confirmed by agarose gel electrophoresis.

RT-PCR Assay

RNA was extracted from 100 μl of serum using SepaGene RV kit (Sanko Junyaku Co., Ltd., Tokyo, Japan). The RNA pellets were resuspended in 10 μl of water containing 10 mM dithiothreitol, 100 pmol random primer (TaKaRa), and 1 unit of RNasin (Promega). The RNA solution was then heated at 65°C for 5 min, chilled on ice, and used immediately. Synthesis of cDNA was carried out in a 20 μl reaction mixture containing 50 mM Tris-HCl(pH 8.3), 75 mM KCl, 3 mM MgCl2, 5 mM DTT, 0.5 mM of each dNTPs, and 200 units of M-MLV RT (GIBCO) at 37°C for 50 min.

For amplification of cDNA by PCR with primer sets I and II, the first round of PCR was performed in a reaction volume of 50 μl containing 10 mM Tris-HCl(pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each of the four dNTPs, 0.2 μM of the outer sense and antisense primers, 1 unit of Taq polymerase (TaKaRa), and 5 μl of cDNA mixture. The reaction was performed for 30 cycles in the thermocycler PE2400 with denaturation at 94°C for 15 sec, primer annealing at 48–55°C for 15 sec according to the Tm of the selected primers, and extension at 72°C for 65 sec. For the second round of PCR, 1 μl of the first PCR reaction was reamplified as described above, except for the substitution of inner sense and antisense primers as well as primer annealing temperature (55°C). Finally, 5 μl of the PCR prod-

TABLE II. Synthetic Oligonucleotide Sequences of HGV

Primer set	Primer name	Oligonucleotide sequences (5'–3')[a]	Nucleotide position (5'–3')[b]
I			
Outer			
sense	sw	GACAGGGTTGGTAGGTCGTAAA	126 to 147
antisense	awm	CCCACTGGTCCTTGTCAACT	318 to 400
Inner			
sense	sn	GTTGGTAGGTCGTAAATCCCG	132 to 152
antisense	an	AGAGAGACATTGAAGGGCGAC	338 to 358
II			
Outer			
sense	G8	TATGGGCATGGHATHCCYCT	4278 to 4297
antisense	G9	TCYTTGATGATDGAACTGTC	4416 to 4435
Inner			
sense	G8	TATGGGCATGGHATHCCYCT	4278 to 4297
antisense	G11	TCYTTACCCCTRTAATAGGC	4398 to 4417
III			
sense	sNS5	TTCTGCTCCACTTGGCTCGCTGAGT	8433 to 8457
antisense	aNS5	GAGGGCCACGATGATGTTAG	8713 to 8732

[a]H denotes A, C, or T; D denotes A, G, or T; Y denotes T or G; and R denotes A or G.
[b]Nucleotide position is based on HGV number system (U44402).

uct was analyzed by electrophoresis on a 2% agarose gel with ethidium bromide staining, and visualized under ultraviolet light.

For amplification of cDNA fragment with primer set III, only a single round of PCR was performed. 5 μl of cDNA synthesis mixture was amplified in the same manner as that in the second PCR reaction described above, except for the choice of the primer and the cycle numbers (40 instead of 30 cycles).

To reduce the risk of contamination in PCR assay, a number of precautions were taken [Kwok et al., 1989]. A negative control was included in every test to monitor the contamination.

To verify the specificity of the PCR analysis, all positive samples were repeated by two individuals, and all amplified products that were generated from the three PCR assays were directly sequenced by fluorescent dye terminator cycle sequencing method using an ABI DNA sequencer 373A (Applied Biosystems, Foster City, CA) as described by Shao et al. [1996].

RESULTS

Selection of Primer Sets

By comparing the available sequences of GBV-C/HGV to known members of *Flaviviridae,* several domains were found in the 5′NC and NS5 regions that were highly conserved among the known GBV-C/HGV strains but shared no significant homology with other members of *Flaviviridae.* Considering this information, the other principles of primer selection, primer sets I and III were chosen for this study. The NS3-derived primer set II which was initially described by Yoshiba et al. [1995] and used widely in Japan was also used for comparison.

Effectiveness of Three Primer Sets for HGV Detection in RT-PCR

To assess the relative sensitivity of these three primer sets, we attempted to amplify each of the 10-fold serial dilution of in vitro synthesized HGV RNA template. As shown in Figure 1, the minimal numbers of HGV RNA copies needed for detection after RT-PCR were about 20 with all three primer sets (I, II, and III).

To test the ability of primer sets, I to III, for detecting HGV RNA in sera of patients, we examined the sera from 371 patients with various hepatitis for the presence of HGV RNA by RT-PCR with each of the primer sets. As summarized in Table III, 21 of the 371 samples were positive with primer set I, whereas 19 and 14 were positive with primer set II, and III, respectively. The difference between sets I and III was statistically significant at the $P < 0.05$ level, although the difference between sets I and II was not. It should be noted that all samples that were positive with primer sets II and III were also positive with primer set I (data not shown). These results suggest that the primer set I is the most effective in detecting HGV RNA.

Prevalence of HGV Infection in Japanese Patients With Various Hepatitis

Table I summarizes the prevalence of HGV infection among the patients with various hepatitis as determined by RT-PCR with primer set I. It is interesting to note that only two out of the 126 patients (1.6%) with non-A-E hepatitis were positive for HGV RNA, with one positive case of fulminant hepatitis. In contrast, HGV RNA were detected more frequently in the patients with hepatitis B and hepatitis C, with positive rates of 5.3% to 8.1%, respectively. Thus HGV infection is relatively common in Japanese patients with hepatitis B and hepatitis C but not in those with non-A-E hepatitis.

DISCUSSION

In this study, we compared the sensitivity of RT-PCR assay for detection of HGV using synthetic viral RNA among the three primer sets derived from the 5′NC, NS3, and NS5 region of HGV genome, respectively, and

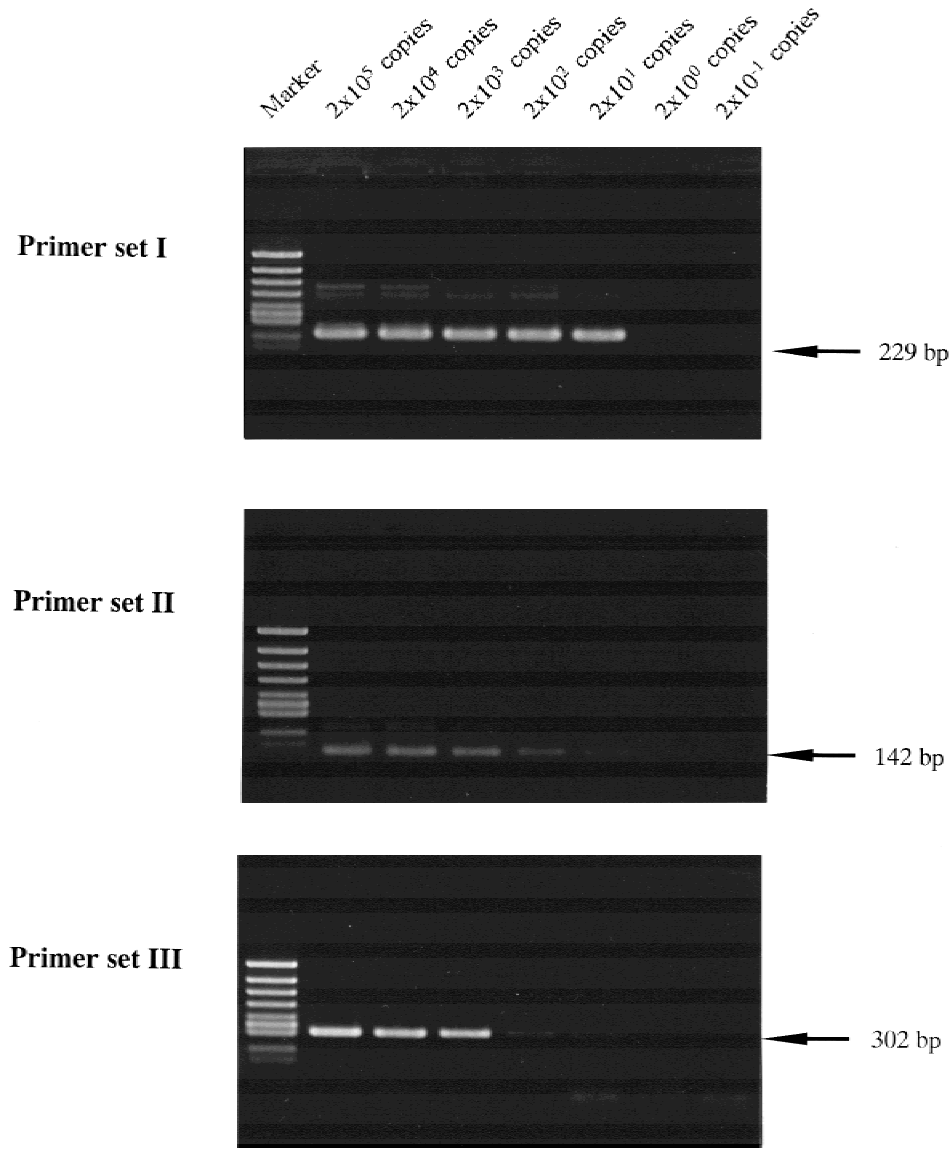

Fig. 1. Relative sensitivity for HGV RNA detection by RT-PCR with the primer sets I–III. The synthetic HGV RNA was initially quantitated and 10-fold serially diluted with DEPC-treated water from 2×10^5 to 2×10^{-1} particles. Each dilution was then used for cDNA synthesis and PCR amplification with the primer sets I–III, respectively. Marker: ΦX174/Hinc II digest; the arrow indicates the size of each amplified DNA band.

TABLE III. Detection of HGV RNA in Sera of the Patients With Various Hepatitis by RT-PCR Using Three Primer Sets

		Number of HGV positive with primer set (%)		
Diagnosis	Number	I	II	III
Non-A-E hepatitis	126	2 (1.6)	2 (1.6)	2 (1.6)
Hepatitis B	38	2 (5.3)	1 (2.6)	1 (2.6)
Hepatitis C	207	17 (8.2)	16 (7.7)	11 (5.3)
Total	371	21 (5.7)	19 (5.1)	14 (3.8)

also attempted to detect HGV RNA in the sera of 371 Japanese patients with hepatitis B, hepatitis C, and non-A-E hepatitis by using these three primer sets. The data showed that all three primer sets could detect approximately equal amount of HGV RNA in RT-PCR assay when the synthetic viral RNA was used as template. The HGV positive rates in the sera of the patients were however different among the primer sets. One possible explanation for this difference is that the genetic heterogeneity of HGV influences the sensitivity and specificity of the tests using different primer sets, because our previous study in addition to others reports revealed that 5′NC, NS3, and NS5 regions of HGV genome exhibit various degrees of sequence divergence, ranging from approximately 11 to 17% among different isolates [Erker et al., 1996; Shao et al., 1996]. Careful attention to the choice of primer from the most conserved region of HGV genome was taken in our study for the optimal detection of serum HGV

RNA. The published sequences of GBV-C/HGV, either complete or partial, are very limited at the present time. By analyzing several GBV-C/HGV sequences that are available [Erker et al., 1996; Fukushi et al., 1996; Leary et al., 1996b; Linnen et al., 1996; Shao et al., 1996] in addition to our own sequence data [unpublished data], as well as the sequences of other members of *Flaviviridae,* we selected three primer sets (I, II, and III) derived from the 5′NC, NS3, and NS5 regions of HGV genome for RT-PCR assay. The result revealed that primer set III detected HGV RNA in only 67% of the sera positive for HGV RNA with primer set I, suggesting that there may be a high rate of false negative results in the assay with primer set III, presumably due to primer and template mismatch caused by the genetic heterogeneity of HGV. Although primer set II was nearly as effective as set I in detecting HGV RNA, BLAST and FLAST searches of GenBank and DDBJ database showed that this primer region shares considerable similarity with the corresponding regions of some HCV strains. Hence, this primer set does not seem to be ideal for detecting HGV RNA. Since all the samples positive for HGV RNA with primer sets II and III were also positive with primer set I, we believe that the primer set I derived from the 5′NC region of the HGV genome is the most effective in detecting HGV RNA. It should be noted here that even as we were preparing this article, comparable observation has been reported by Muerhoff and coworkers [1996]. Although their study did not ascertain whether all of the selected primer sets can detect an equivalent amount of HGV RNA in a standard PCR approach, based on comparison of the primer sets from 5′NC and NS3 regions, the results also suggest that certain 5′NC-derived primers appear to be most efficient for virus detection.

Besides the comparison of primer sets, we have elucidated the prevalence of HGV infection in Japanese patients with hepatitis B, hepatitis C, and non-A-E hepatitis. It was found that only two out of the 126 patients (1.6%) with non-A-E hepatitis were positive for HGV RNA. This value is lower than those reported in other countries (8.2–39%) [Fiordalisi et al., 1996; Linnen et al., 1996]. Because the observation was based on the RT-PCR with multiple primer sets in which as few as 20 HGV RNA copies could be detected, we believe that HGV, at least in Japan, accounts for only a minority of the cases of non-A-E hepatitis. Furthermore, it is reasonable to conclude that there are differences in the prevalence of HGV between the geographically different areas. The positive rate of HGV RNA in our patients with non-A-E fulminat hepatitis was also low (12.5%) compared with the rate (37.5%) reported by Yoshiba et al. [1996]. However, caution must be taken in interpreting such data since the cases with non-A-E fulminant hepatitis in our study were limited in number. Taken together with other report which show HGV positive in three out of 25 Japanese patients (12%) with non-A-E fulminant hepatitis [Tameda et al., 1996], the data raised the alternative possibility that HGV may not be the common cause of non-A-E fulminant hepatitis in Japan, although the possibility that particular virulent HGV strains prevalent in some areas of Japan that cause fulminant hepatitis could not be ruled out. Thus, the significance of HGV in the etiology of cryptogenic fulminant hepatitis should be further investigated. In addition, our data further confirm the previous findings that HGV RNA was detected more frequently in patients with chronic hepatitis B and chronic hepatitis C [Schleicher et al., 1996; Schreier et al., 1996; Tanaka et al., 1996; Wang et al., 1996].

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